Loss of Calcineurin Aα Alters Keratinocyte Survival and Differentiation

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Calcineurin is a serine/threonine phosphatase that is inhibited by the immunosuppressive drugs cyclosporine and FK506. Although calcineurin has been extensively studied in immune cells, less is known about calcineurin in other systems. There are two primary isoforms of the catalytic subunit of calcineurin, and mice have been created that lack either the α isoform (calcineurin A (CnA)$\alpha^{-/-}$) or the β isoform (CnA$\beta^{-/-}$). In this study, we examined the epidermis of CnA$\alpha^{-/-}$ mice at birth and 4 weeks of age. Histological analyses revealed an attenuation of cells in the stratum spinosum of CnA$\alpha^{-/-}$ mice. There was no significant difference in proliferation in the epidermis of CnA$\alpha^{-/-}$ sections, but TUNEL assay revealed increased cell death in the supra-basal layers. Interestingly, the calcineurin substrate nuclear factor of activated T cells (NFATc) was highly expressed in the nucleus of basal epidermal cells in wild-type (WT) mice but was cytoplasmic in CnA$\alpha^{-/-}$ mice, consistent with a loss of calcineurin activity. Moreover, NFATc activity was decreased in the epidermis of null mice compared with that in WT littermates. Finally, immunohistochemical staining revealed supra-basal expression of keratin 14 and decreased expression of differentiation-associated keratin 10 and involucrin. These findings suggest that calcineurin Aα activity is required for the normal differentiation and survival of epidermal cells.

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INTRODUCTION

Calcineurin is a serine/threonine protein phosphatase that is essential for coordinating calcium signaling with nuclear action. It was initially localized to neural tissue but has since been described in other tissues, most notably the immune system, in which inhibition of calcineurin by cyclosporine leads to a loss of T-cell function (Rusnak and Mertz, 2000). Calcineurin modulates the transcription factor nuclear factor of activated T cells (NFATc), which is cytoplasmic and inactive when phosphorylated. Calcium-dependent dephosphorylation by calcineurin leads to nuclear translocation and modification of target genes including IL-2 (Liu *et al.*, 1991; Rao *et al.*, 1997).

There are three isoforms of the catalytic subunit of calcineurin, namely α, β, and γ. The α and β isoforms are found in most mammalian tissues, whereas γ is primarily expressed in the testes and brain (Rusnak and Mertz, 2000). Mice lacking the α and β isoforms have been created and have been reported to have distinct phenotypes. For example, calcineurin A (CnA)$\beta^{-/-}$ mice are viable, fertile, and overtly normal well into adulthood, but have abnormal immune systems (Bueno *et al.*, 2002). In contrast, CnA$\alpha^{-/-}$ mice are significantly smaller, infertile, and typically only live a few weeks (Zhang *et al.*, 1996; Zhuo *et al.*, 1999; Gooch *et al.*, 2004). Their immune systems, however, are only mildly affected and the mice can still be immunosuppressed by cyclosporine (Zhang *et al.*, 1996). Moreover, CnA$\alpha^{-/-}$ mice have defects in renal development and function including increased fibrosis and upregulation of TGFβ (Gooch *et al.*, 2007). Cyclosporine and FK506 are inhibitors of calcineurin that have been used to treat a number of disorders involving multiple organ systems. They have revolutionized transplant medicine and have also been useful in the treatment of many skin disorders (Lim *et al.*, 1996). The effects of cyclosporine on dermatological conditions are typically attributed to suppression of epidermal immune cells. However, a direct role for calcineurin in the epidermis cannot be excluded.

For example, calcineurin activity has been recently measured directly in cells that populate skin, including melanocytes and keratinocytes (Smit *et al.*, 2008). Moreover, inhibition of calcineurin has been shown to block proliferation of cultured keratinocytes (Fisher *et al.*, 1988; Nickoloff *et al.*, 1988). Others have noted that suppression of calcineurin inhibits p21 expression and downregulates keratinocyte differentiation (Santini *et al.*, 2001). These results bear some similarities to findings reported by our

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Abbreviations: CnAα, calcineurin Aα; NFATc, nuclear factor of activated T cells; WT, wild-type

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laboratory. We have previously shown changes in proliferation and expression of the cyclin-dependent kinase inhibitor p27 with loss of CnAα in the kidney (Gooch *et al.*, 2004). This suggests that the α isoform of calcineurin may be involved in pathways that are also important for normal differentiation and/or cell cycle control of epidermal cells. More importantly, other authors have connected the Notch signaling cascade with the calcineurin/NFAT pathway (Mammucari *et al.*, 2005). This finding is significant, as Notch signaling has been shown to be a key player in the progression of keratinocytes from basal to spinosum and granular layers (Moriyama *et al.*, 2008). Therefore, we examined the skin of newborn and 4-week-old CnAα$^{-/-}$ mice and wild-type (WT) littermates, and reported that CnAα plays a direct role in the differentiation and survival of keratinocytes.

RESULTS

Mice lacking CnAα show skin abnormalities

To examine a potential role for calcineurin in the epidermis, CnAα$^{-/-}$ and WT epidermal sections were collected and stained using H&E, and examined by light microscopy. At birth, WT epidermal sections consisted of a well-organized basal-cell layer, two- to three-cell thickness of stratum spinosum, and normal appearing granular and corneum layers (Figure 1a). In contrast, the stratum spinosum of newborn CnAα$^{-/-}$ mice seemed markedly attenuated (Arrowhead, Figure 1b). Although the granular layer seems unremarkable, there is a thickening of the corneum and a greater number of keratinocytes seem to be in the process of ''sloughing-off'' (Figure 1b). At 4 weeks of age, despite normal follicular eruption, a similar attenuation of the epidermis is observed in CnAα$^{-/-}$ mice (Figures 1c and d). Skin elasticity was observed by comparing the response of 4-week-old CnAα$^{-/-}$ and WT littermates to deformation of the nape by pinching. Results were noted at the time WT skin returned to a normal appearance. Deformation persisted longer in CnAα$^{-/-}$ than was observed in WT mice (Figure 1e). In addition, epidermal sloughing in arm and neck folds was observed in CnAα$^{-/-}$ pups before follicular eruption (data not shown). Sections from CnAβ$^{-/-}$ samples were consistent with the normal appearance observed in WT samples, suggesting that the α isoform plays a predominant role in epidermis (Supplementary Figure S1).

Loss of CnAα increases apoptosis and results in alterations in keratin expression

Changes in CnAα$^{-/-}$ epidermal sections were further characterized. To begin, cell proliferation within the epidermis of WT and CnAα$^{-/-}$ mice was determined by identifying cells that express the DNA replication protein proliferating cell nuclear antigen. Similar to the WT, the majority of proliferating cells were identified in the stratum basale and in the developing follicles of CnAα$^{-/-}$ sections (Figures 2a and b). Quantitation of results from WT and CnAα$^{-/-}$ mice showed that there were no significant differences in proliferation with loss of CnAα (Figure 2e). In addition to proliferation, cell death in the epidermis of WT and CnAα$^{-/-}$ sections was examined by TUNEL stain. This technique revealed an increase in cells undergoing cell death in the supra-basal layers of CnAα$^{-/-}$ sections compared with that in WT (Figure 2c and d), consistent with attenuation of the stratum spinosum layer observed by H&E stain (Figure 1b). Quantitation of results from WT and CnAα KO mice revealed a significant increase in cell death in the stratum spinosum with loss of CnAα (Figure 2f).

Calcineurin activity *in vivo* can be indirectly followed by subcellular localization of target proteins including NFATc, which translocate to the nucleus on dephosphorylation by calcineurin. Therefore, subcellular localization of NFATc was examined by immunofluorescence in WT and CnAα$^{-/-}$ epidermal sections. Figure 3a shows that NFATc is readily detectable in basal epidermal cells and that it is predominantly nuclear (asterisk, Figure 3a). In contrast, NFATc seemed to be excluded from the nucleus in CnAα$^{-/-}$ mice (arrow, Figure 3b). Cytoplasmic localization suggests a loss of NFATc transcriptional activity. To examine NFATc activity,

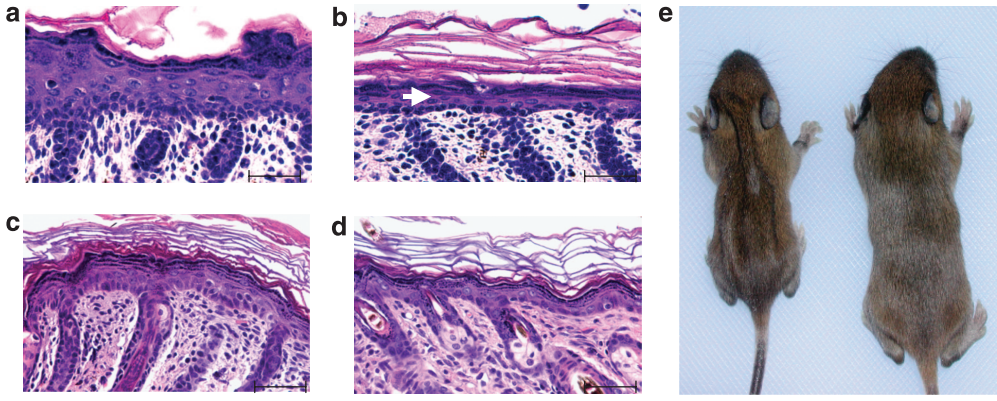

Figure 1. Changes in the skin with loss of CnAα. Epidermal sections from newborn WT (**a**) and CnAα$^{-/-}$ (**b**) mice were obtained from the nape of the neck, paraffin-embedded, stained with H&E, and examined by light microscopy. Arrowhead indicates attenuation of the stratum spinosum. Results shown are representative of at least five mice per genotype. Epidermal sections were then likewise obtained from 4-week-old WT (**c**) and CnAα$^{-/-}$ (**d**) mice. (**e**) The nape of WT and CnAα$^{-/-}$ mice was gently pinched for 5 seconds and then a return to normal appearance was observed. Deformation of CnAα$^{-/-}$ mice (left) was still apparent by the time WT mice (right) had returned to normal. Data shown is typical result of multiple trials using five mice per genotype. Bar = 20 μm.

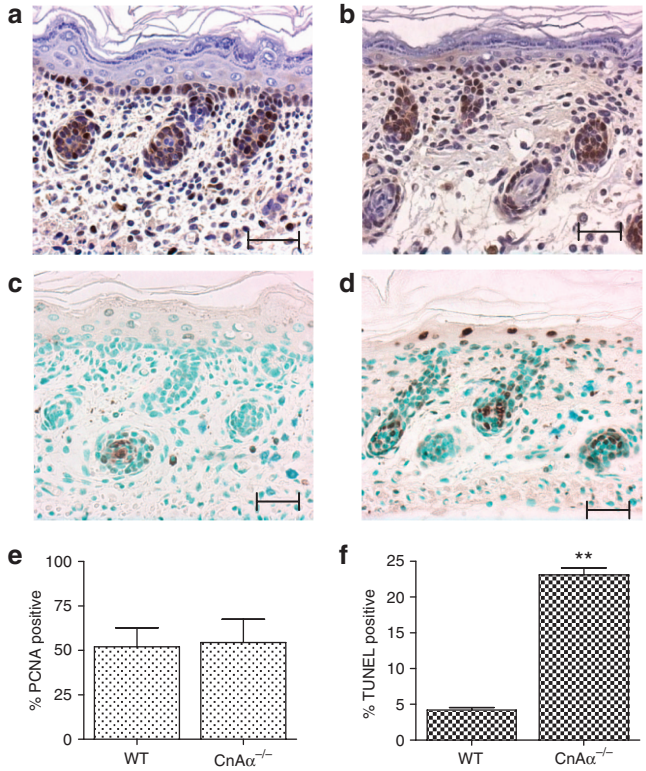

Figure 2. Changes in cell cycle with loss of CnAα$^{-/-}$. Proliferating cells within the epidermis were identified in newborn WT (**a**) and CnAα$^{-/-}$ (**b**) epidermal sections by expression of the DNA replication protein proliferating cell nuclear antigen (PCNA) after immunohistochemistry using a specific antibody. Results are typical for at least three mice per genotype. Bar = 50 μm. Cell death within the epidermis was determined in wild-type (**c**) and CnAα$^{-/-}$ mice (**d**) by TUNEL assay. Data presented are representative of results from at least three different mice per group. Bar = 50 μm. (**e**) PCNA expression as shown in panels **a** and **b** was semi-quantitated and graphed. Positive cells in at least three separate frames (20–40 cells per frame) were scored by a blinded reviewer for each animal. Data shown are the mean ± SEM of at least three different mice per genotype. No significant difference in cell proliferation was noted between the WT and CnAα$^{-/-}$ sections. (**f**) Cell death indicated by TUNEL assay was semi-quantitated and graphed. Positive cells in at least three separate frames (20–40 cells per frame) were scored by a blinded reviewer for each animal. Results from at least three different animals were semi-quantitated and graphed. Data shown are the mean ± SEM. There were significantly more cells undergoing apoptosis in CnAα$^{-/-}$ sections than in WT (**$P<0.01$).

skin samples were obtained from WT and CnAα$^{-/-}$ mice that also express an NFATc-driven luciferase reporter construct (Parsons *et al.*, 2003). Epidermal samples were minced, incubated in lysis buffer, and then luciferase expression was quantified by luminescence. Figure 3c shows that there is a significant decrease of 74% ± 2.2 (SEM) in luciferase activity in CnAα$^{-/-}$ epidermal sections compared with that in wild type.

Finally, we examined expression of proteins that are specific for the basal, spinosum, and granular layers. In WT sections, there is robust expression of keratin 14 in the basal layer (Figure 3d), whereas the fully differentiated supra-basal layers express keratin 10 (Figure 3e) and the granular layer expresses involucrin (Figure 3f). This arrangement was not found in CnAα$^{-/-}$ mice. Instead, supra-basal layers continue to express keratin 14 (Figure 3g, arrowhead) and show attenuation of keratin 10 (Figure 3h, arrow) and involucrin (Figure 3i). These findings suggest that loss of CnAα leads to impaired differentiation of epidermal keratinocytes.

DISCUSSION

In this study, we have shown that loss of the α isoform of calcineurin leads to abnormal differentiation of keratinocytes and an altered epidermis. Grossly, the skin of CnAα$^{-/-}$ mice is mildly abnormal and characterized by increased ''laxness'' and decreased elasticity. Histologically, the normal architecture found in WT dermis is altered. There is an attenuation of the supra-basal layers that seems to be due to increased apoptosis and/or premature differentiation of keratinocyte. Moreover, the deletion of calcineurin leads to loss of nuclear NFATc localization in basal cells and decreased NFATc activity in the epidermis. Finally, loss of calcineurin is associated with defects in the differentiation of keratinocytes. Supra-basal layers retain expression of keratin 14, and express less keratin 10 and involucrin. Taken together, these findings support a role for the α isoform of calcineurin in normal differentiation and survival of keratinocytes. The absence of similar changes in CnAβ$^{-/-}$ mice suggests that keratinocyte survival and differentiation may be additional examples of isoform-specific actions of CnAα.

Although previous studies have found that loss of calcineurin activity in the epidermis alters follicular cells and hair growth (Al-Daraji *et al.*, 2002; Gafter-Gvili *et al.*, 2003), it is interesting to note that CnAα$^{-/-}$ mice have no observable hair growth or pigmentation defects through 4 weeks of age. This may be due, however, to a lack of data from older animals due to the early death of null mice. Despite the limitations of the model, CnAα$^{-/-}$ mice may offer insight not only into the skin changes associated with calcineurin inhibitors, but also dermatological disorders such as psoriasis.

The relationship between calcineurin and immunomodulation has been well established, and calcineurin inhibition has found a central role in the treatment of dermatological pathologies. The therapeutic effects of these drugs have been attributed to the immunomodulatory effect of calcineurin inhibitors. However, there is evidence that other mechanisms are in play. A recent study in this journal measured calcineurin activity in the skin, and successfully showed cyclosporine-mediated calcineurin inhibition in keratinocytes and melanocytes (Smit *et al.*, 2008). Cyclosporine has been shown *in vitro* to alter proliferation of keratinocytes (Fisher *et al.*, 1988; Nickoloff *et al.*, 1988), and multiple reports have linked calcium and calcium-dependent signaling mechanisms to keratinocyte differentiation (Yuspa *et al.*, 1989; Santini *et al.*, 2001; Mammucari *et al.*, 2005; Sakaguchi *et al.*, 2005). More specifically, calcineurin and NFATc are expressed in the epidermis (Al-Daraji *et al.*, 2002), and activation of the pathway is linked to control of cell cycle proteins including p21 (Santini *et al.*, 2001; Mammucari

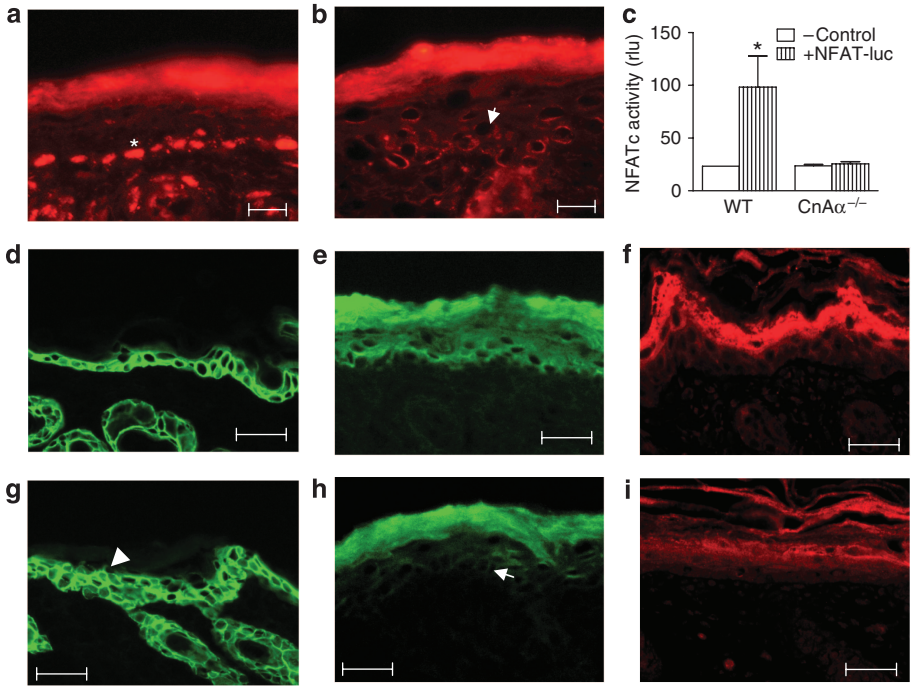

Figure 3. NFATc activity is lost and differentiation is altered with loss of CnAα. Cellular localization of the calcineurin substrate NFATc was determined by immunofluorescence in epidermal sections from 4-week-old wild-type (**a**) and CnAα$^{-/-}$ (**b**) mice using an antibody that recognizes all calcineurin-regulated NFATc proteins. Protein expression was visualized using a Cy3-conjugated secondary antibody. Bar = 20 μm. Data shown are representative of at least three different mice per genotype. Asterisk indicates nuclear localization in WT and arrowhead indicates cytoplasmic localization in CnAα$^{-/-}$ (**b**). (**c**) NFATc activity was examined in dermal/epidermal skin samples from 4-week-old wild-type and CnAα$^{-/-}$ mice that constitutively express an NFATc-responsive luciferase promoter construct. Samples were obtained from mice positive for the reporter transgene, and luciferase activity was measured. Results were compared with background luminescence values obtained from samples from littermate mice lacking the reporter construct (control). Data shown are the mean ± SEM of three mice per group. Next, expression of the epidermal markers were examined in 4-week-old WT mice including K14 (**d**), K10 (**e**), and involucrin (**f**) by immunofluorescence. Protein expression was visualized using FITC-conjugated or Cy3-conjugated secondary antibodies. Data shown are representative of at least three mice per group. Finally, the same markers were examined in epidermal sections from 4-week-old CnAα$^{-/-}$ mice (**g–i**). The arrowhead highlights supra-basal expression of K14 and the arrow indicates lack of expected K10 expression in supra-basal layers. Data shown are representative of at least three mice per group.

et al., 2005). Finally, calcineurin has been connected to the Notch signaling cascade (Mammucari *et al.*, 2005), a significant finding given Notch's central role in coordinating epidermal development (Moriyama *et al.*, 2008). Moreover, the Notch cascade is also important in certain disease processes, including psoriasis (Thelu, 2002). Our data showing specific changes in keratinocyte differentiation and survival with loss of CnAα provides yet more evidence that calcineurin is an important component of the epidermis. The phenotype we report in the CnAα$^{-/-}$ mouse model is particularly significant in light of previous findings that the immune system of null mice develop normally (Zhang *et al.*, 1996) and that mice with CnAα$^{-/-}$ immune systems can still be immunosuppressed with cyclosporine (Zhang *et al.*, 1996). Therefore, a role for calcineurin in epidermal differentiation and survival may be independent of its action in the immune system.

Further, psoriasis is a common skin disorder that is caused by an immune reaction including inflammatory cytokine production and characterized by hyper-proliferation of the stratum spinosum (Chamian and Krueger, 2004). The immunological angle of the disease process has been well established, and is supported by the therapeutic benefits of cyclosporine. However, it is possible that the benefits of cyclosporine treatment may also be due to a direct effect on the cellular machinery of keratinocytes. Interestingly, Notch genes seem to play a role in psoriasis, as these have been noted to be downregulated in psoriatic plaques (Thelu, 2002). We have shown here that loss of CnAα leads to a phenotype that is the opposite of psoriasis—increased cell death in the stratum spinosum and fragile skin. Furthermore, the therapeutic success of cyclosporine to heal psoriatic plaques may also be due to its ability to alter differentiation of keratinocytes and survival of supra-basal cells.

We have presented data, to our knowledge previously unreported, that supports the hypothesis that calcineurin is required for the differentiation and survival of keratinocytes. CnAα$^{-/-}$ mice are characterized by increased skin laxness, with epidermal sections that show an attenuation of the stratum spinosum and decreased NFATc activity. Moreover, CnAα$^{-/-}$ stratum spinosum keratinocytes fail to differentiate normally and undergo cell death. These findings provide further evidence of the role of calcineurin keratinocytes differentiation and survival.

MATERIALS AND METHODS

Materials

Antibodies that recognize all calcium-dependent isoforms of NFAT (c1-c4), and keratins 10 and 14 were obtained from Santa Cruz (Santa Cruz, CA); anti-proliferating cell nuclear antigen antibody was obtained from Dako (Glostrup, Denmark).

Animal models

CnAα$^{-/-}$ mice were created by J. Seidman (Howard Hughes Medical Institute, Harvard Medical School, Boston, MA) as previously described (Zhang *et al.*, 1996) and kindly provided to our laboratory. Animals were maintained and bred in the animal facility at the Atlanta Veteran Affairs Medical Center, Decatur, GA, in accordance with Institutional Animals Care and Use Committee standards. Genotypes of offspring from heterozygous breeding pairs were determined by PCR reaction using the following primers: 5′-GGCAGGAGAGTAAATTCTTGC, 3′-GTGGAATTCTCTGGAGA-CAAACCACC, and neo-TCTTGATTCCCACTTTGTGGTTCTA. CnAβ mice were created by J. Molkentin (Cincinnati Children's Hospital, Cincinnati, OH) and were previously described (Parsons *et al.*, 2003). CnAα$^{-/-}$ mice were created on a mixed genetic background. Therefore, all the experiments were carried out with WT and, where indicated, heterozygous littermates.

NFATc-driven luciferase reporter mice

Mice expressing an NFATc-responsive luciferase reporter plasmid (*NFATc-luc*) were created by Jeffrey Molkentin (Cincinnati Children's Hospital, Cincinnati, OH) and were previously described (Parsons *et al.*, 2003). Mice expressing one copy of the NFATc-luciferase plasmid were bred with CnAα$^{+/-}$ mice to produce WT and CnAα$^{-/-}$ mice that express the reporter plasmid as well as littermates that are negative for *NFATc-luc*, which serve as negative controls. Skin samples including both dermis and epidermis were harvested from mice, finely minced, and then lysed in 1 µl of passive lysis buffer per milligrams of tissue. Luciferase expression was then measured according to the manufacturer's instructions (Promega, Madison, WI) and quantified using a luminometer. As an internal control for background luminescence, luminescence values were also obtained from littermate mice that do not express the construct.

Histology

Epidermal sections from newborn mice were immersed in formalin or quickly frozen in liquid nitrogen for further analyses. Routine histology was on 4-µm thick sections from each genotype stained with H&E. In addition, epidermal sections from three to five animals of each genotype were analyzed using TUNEL assay to identify cells undergoing cell death.

Immunohistochemistry. It was performed as previously described (Gooch *et al.*, 2004). Sections were counter-stained with hemotoxylin, coverslips were mounted with Permount (Sigma, St Louis, MO), and sections were viewed by brightfield microscopy.

Immunofluorescence. Frozen sections of 6-µm thickness were mounted on glass slides and then fixed in acetone. Sections were rehydrated in PBS (phosphate-buffered saline)/0.1%BSA before blocking with the appropriate IgG. Primary antibodies were added at concentrations between 10 and 20 µg ml^{-1} for 1 h at room temperature. After incubation with primary antibodies, sections were washed thrice for 5 minutes each time in PBS/0.1%BSA. Fluorescence-conjugated secondary antibodies were added at dilutions of 1:100 for 45 minutes at room temperature followed by washing in PBS/0.1%BSA. Sections were mounted with Crystal Mount (Dako) and allowed to dry before viewing with fluorescence microscopy.

CONFLICT OF INTEREST

The authors state no conflict of interest.

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SUPPLEMENTARY MATERIAL

Supplementary material is linked to the online version of the paper at http://www.nature.com/jid

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